Title

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Journal

PLoS genetics, 15(10)

ISSN

1553-7390

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Publication Date

2019-10-01

DOI

10.1371/journal.pgen.1008474

Peer reviewed

PLOS GENETICS

RESEARCH ARTICLE

Splice variants of DOMINO control *Drosophila* circadian behavior and pacemaker neuron maintenance

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Citation: Liu Z, Tabuloc CA, Xue Y, Cai Y, Mcintire P, Niu Y, et al. (2019) Splice variants of DOMINO control *Drosophila* circadian behavior and pacemaker neuron maintenance. PLoS Genet 15 (10): e1008474. https://doi.org/10.1371/journal.pgen.1008474

Editor: Gregory P. Copenhaver, The University of North Carolina at Chapel Hill, UNITED STATES

Received: November 19, 2018

Accepted: October 11, 2019

Published: October 28, 2019

Data Availability Statement: The numerical data and summary statistics are available for download at GitHub (https://github.com/yongzhangclock/domino). All other data are within the manuscript and its Supporting Information files.

Funding: YZ's lab is supported by the National Institutes of Health under grant numbers R15 ES030548, P20 GM103650, GM103554, and GM103440. JCC is supported by NIH R01 GM102225 and NSF IOS 1456297. The funders had no role in study design, data collection and

Abstract

Circadian clocks control daily rhythms in behavior and physiology. In *Drosophila*, the small ventral lateral neurons (sLN_vs) expressing PIGMENT DISPERSING FACTOR (PDF) are the master pacemaker neurons generating locomotor rhythms. Despite the importance of sLN_vs and PDF in circadian behavior, little is known about factors that control sLN_vs maintenance and PDF accumulation. Here, we identify the *Drosophila* SWI2/SNF2 protein DOMINO (DOM) as a key regulator of circadian behavior. Depletion of DOM in circadian neurons eliminates morning anticipatory activity under light dark cycle and impairs behavioral rhythmicity in constant darkness. Interestingly, the two major splice variants of DOM, DOM-A and DOM-B have distinct circadian functions. DOM-A depletion mainly leads to arrhythmic behavior, while DOM-B knockdown lengthens circadian period without affecting the circadian rhythmicity. Both DOM-A and DOM-B bind to the promoter regions of key pacemaker genes *period* and *timeless*, and regulate their protein expression. However, we identify that only DOM-A is required for the maintenance of sLNvs and transcription of *pdf*. Lastly, constitutive activation of PDF-receptor signaling rescued the arrhythmia and period lengthening of DOM downregulation. Taken together, our findings reveal that two splice variants of DOM play distinct roles in circadian rhythms through regulating abundance of pacemaker proteins and sLN_vs maintenance.

Author summary

Circadian rhythms are critical for timing of animal bodily functions. In flies, sLN_vs are the master pacemaker neurons regulating locomotor rhythms, which release the neuropeptide PDF. Little is known about factors that control sLN_vs maintenance and PDF accumulation. Here, we identified the *Drosophila* chromatin remodeler DOMINO (DOM) as a new regulator of circadian behavior. Depletion of DOM in circadian neurons impaired behavioral rhythmicity in constant darkness. Interestingly, two splice variants of DOM have

analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

distinct functions. DOM-A depletion mainly led to arrhythmia, while DOM-B knockdown lengthened circadian period. Furthermore, we found DOM-A is critical for the maintenance of sLN_vs and transcription of *pdf*. Our findings reveal that DOM splice variants play distinct roles in rhythms through different mechanisms.

Introduction

Circadian clocks allow animals to anticipate daily oscillations in behavior, physiology and metabolism [1]. The core of the molecular clock is a negative transcriptional-translational feedback loop, which is evolutionarily conserved across species [2]. The fruit fly *Drosophila melanogaster* has been a powerful model in dissecting the molecular and neuronal mechanisms of circadian rhythms. In *Drosophila*, a heterodimeric complex of CLOCK (CLK) and CYCLE (CYC) activates rhythmic transcription of clock-controlled genes, including the transcriptional repressor *period* (*per*) and *timeless* (*tim*). PER and TIM dimerize and repress their own transcription by blocking CLK-CYC transactivation [3]. A number of kinases (CK1, SGG, NEMO, etc) and phosphatases (PP1, PP2A) also control the circadian clock at the post-translational level [3].

Circadian locomotor rhythms are controlled by a small set of clock neurons expressing core pacemaker proteins in the brain. In each hemisphere of the fly brain, there are ~75 clock neurons, which are divided into clusters based on their anatomical locations and functions in circadian behavior [4]. There are three groups of dorsal neurons (DN1s, DN2s, and DN3s), the lateral dorsal neurons (LNds), the lateral posterior neurons (LPNs), the large ventral lateral neurons (lLN_vs), and the small ventral lateral neurons (sLN_vs). The lLN_vs and four sLN_vs express the neuropeptide PIGMENT DISPERSING FACTOR (PDF), while the fifth sLN_v is PDF negative. The PDF positive sLN_vs are the key pacemaker neurons: they control the circadian rhythmicity under constant darkness (DD) [4]. They are also critical for generating morning anticipatory activity before lights on (i.e. dawn) under light-dark cycle (LD) [5, 6]. The sLN_vs send axonal projections towards the DN1s and DN2s, and the structural plasticity of dorsal projections is under circadian control [7–9]. PDF positive sLN_vs and their dorsal projections are formed in 4 hrs old first instar larvae after hatching [10]. Despite the importance of sLN_vs in the circadian behavior, little is known about the mechanisms that control the maintenance of sLN_vs.

PDF is the most prominent neuropeptide in the regulation of circadian behavioral rhythms [11]. PDF plays a critical role in the synchronization of different groups of clock neurons by binding to PDF receptor (PDFR), and activating cAMP-activated protein kinase A (PKA) signaling [12–15]. Loss of PDF or PDFR abolishes morning anticipation and significantly impairs behavioral rhythmicity in DD. Not only as a critical circadian output signal, PDF/PDFR signaling has also been shown to control the molecular clock by regulating the stability of pacemaker proteins PER and TIM recently [16–18]. PDF abundance in the sLN_vs is regulated by CLK and CYC [19–21]. Another pacemaker protein VRILLE also promotes PDF levels [22]. Despite these studies, mechanisms regulating PDF abundance remain poorly understood.

DOM is a chromatin-remodeling protein, which belongs to the SWI/SNF2 DNA-dependent ATPase family [23]. DOM is in the TIP60 complex and plays a critical role for incorporation of the phosphorylated Histone H2A variant (H2Av) and exchange with H2Av in *Drosophila* [24]. It has been found that DOM is involved in oogenesis, wing development, cell viability and proliferation, neuroblast maintenance and polarity, as well as in dendrite development [23, 25–27]. In the fly genome, two alternative splicing variants of *dom* encode DOM-A

and DOM-B. Interestingly, a recent study identified that DOM-A and DOM-B play distinct roles in cell-type specific development during *Drosophila* oogenesis [25].

A few chromatin remodelers have been shown to regulate circadian photo-responses and gene expression [28–30]. However, the role played by chromatin remodeling in the control of *Drosophila* circadian clocks is still largely unknown. Here, we report the functions of DOM in the regulation of circadian rhythms. Using isoform-specific RNAi and rescue, we demonstrate distinct functions of DOM-A and DOM-B in the regulation of circadian locomotor rhythms. Depletion of DOM-A in *tim*-expressing circadian neurons leads to arrhythmic behavior and long period, while DOM-B downregulation specifically lengthens the circadian period in flies. Both DOM-A and DOM-B bind to the promoters of *per* and *tim*, and regulate the abundance of PER and TIM levels, which is critical for the control of circadian period. However, DOM-A is specifically necessary for maintenance of sLN_vs as well as accumulation of PDF in these neurons. Indeed, activation of PDFR signaling restores the locomotor rhythms of DOM downregulation. Together, our results suggest that the two alternative spliced variants of DOM play distinct roles in circadian rhythms.

Results

DOM regulates circadian locomotor rhythms

Mass spectrometry (MS) label-free quantitative proteomics approach was previously performed to identify the BRAHMA (BRM) chromatin-remodeling protein complex as interactor of circadian clock proteins [31]. In the same data set, we observed that several core subunits of the ATP-dependent DOM chromatin-remodeling complex, including DOM, NIPPED-A, PONT, REPT, and Mrg15, interact with CLK in the nucleus of *Drosophila* S2 cells (Table 1). Since DOM is the ATPase subunit of the protein complex and shows significant binding to CLK (especially C-terminal FLAG-tagged CLK) according to SAINT (Significant Analysis of

Table 1. Subunits of the DOM chromatin-remodeling complex interact with CLK in the nucleus of *Drosophila* Schneider (S2) cells as detected by FLAG affinity purification followed by mass spectrometry.

		Bait: N-Terminus tagged CLK				Bait: C-Terminus tagged CLK				Control AP-MS
Symbol	FlyBaseID	peptide[a] Count(3 reps)	AvgP[b]	MaxP[c]	False[d] Discovery Rate (FDR)	peptide[a] Count(3 reps)	AvgP[b]	MaxP[c]	False[d] Discovery Rate (FDR)	Control[e] Count (4)
Domino	FBgn0020306	37|0|46[f]	0.6526	0.9825	0.0663	30|17|16	1	1	0	0|0|0|0[g]
Nipped-A	FBgn0053554	53|21|114	1	1	0	75|45|55	1	1	0	0|0|0|0
His2AV	FBgn0001197	7|1|8	0.8631	0.9896	0.0172	1|0|3	0.5466	0.8827	0.1643	0|0|0|0
Pont	FBgn0040078	27|15|34	1	1	0	25|22|26	1	1	0	0|0|0|0
Rept	FBgn0040075	28|16|30	1	1	0	30|9|14	0.9994	1	0.0001	2|0|1|0
E(Pc)	FBgn0000581	17|0|20	0.6398	0.9638	0.1001	34|17|17	1	1	0	0|0|0|0
Mrg15	FBgn0027378	4|3|2	0.9874	0.9937	0.0010	6|0|3	0.6367	0.9718	0.0875	0|0|0|0

[a] Number of peptides mapped to the specified prey protein in affinity purification (AP) followed by mass spectrometry (MS).

[b] Average probability that protein interactions between the bait and prey protein are *bona fide*. Values were from all biological replicated as calculated by SAINT (Significant Analysis of Interactome).

[c] Highest probability of protein interaction between the bait and prey protein across all replicate as calculated by SAINT.

[d] False discovery rate as calculated by SAINT using all biological replicates.

[e] Number of peptides mapped to prey protein in control AP-MS.

[f] Peptide counts for 3 biological replicates are shown.

[g] peptide counts for 4 biological replicates are shown.

https://doi.org/10.1371/journal.pgen.1008474.t001

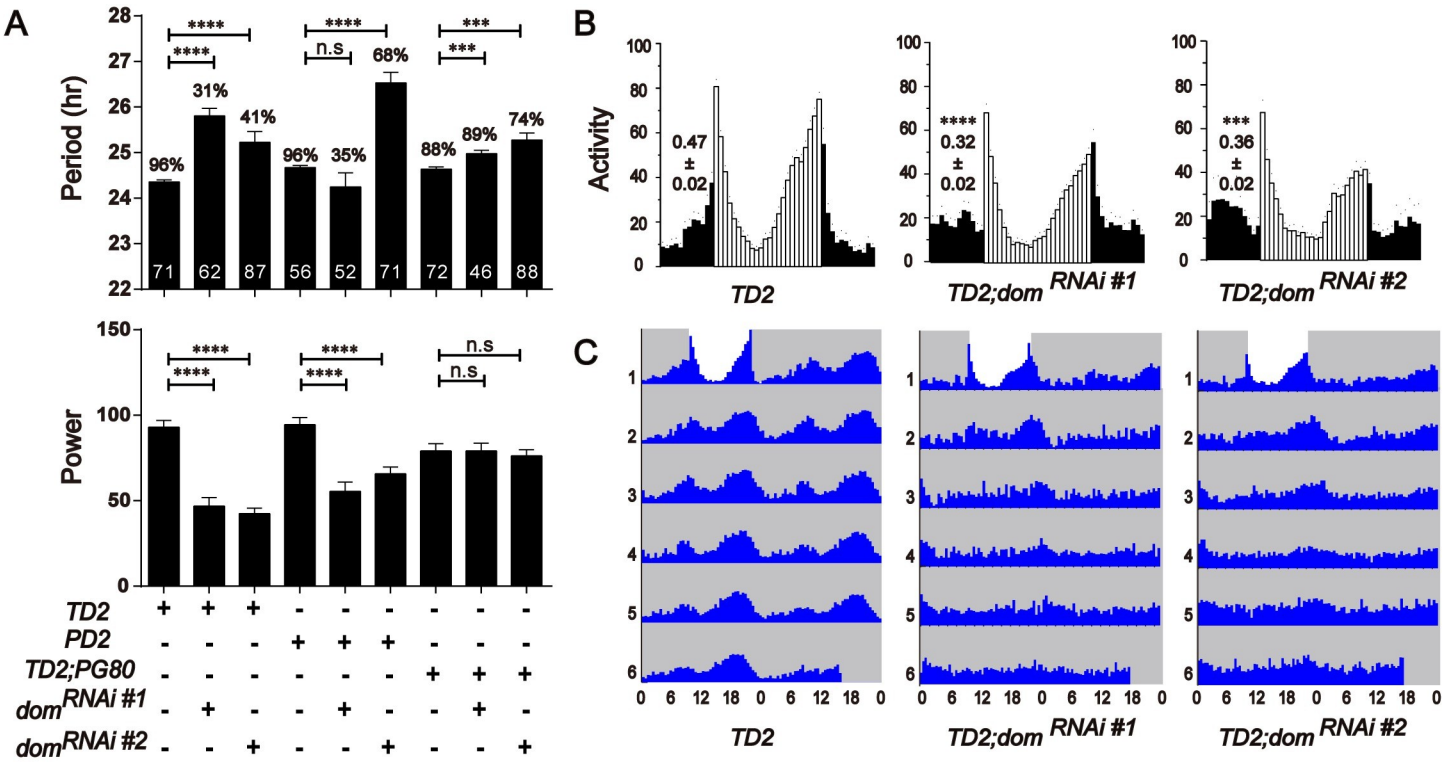

Fig 1. Depletion of DOM disrupted circadian rhythms. A. Free-running period (top panel) and power (bottom panel) of DOM depleted flies. The percentages of rhythmic flies are shown above each column. The number of tested flies is shown in each column. *dom* mRNA targeted by the two non-overlapping RNAi lines from the TRiP stock center. $dom^{RNAi\#1}$ (JF01502) is a long double-stranded RNAi line, while $dom^{RNAi\#2}$ (HMS01855) is a short hairpin RNAi line. TD2 = *tim-GAL4*, *UAS-dcr2*, PD2 = *pdf-GAL4*, *UAS-dcr2*, TD2; PG80 = *TD2*, *pdf-GAL80*. n = 50–90; error bars represent ± SD; n.s., non-significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$; one-way ANOVA. **B.** Average locomotor activity of flies under 3 days of 12:12 hr LD conditions. Dark activity bars represent the night, and white bars represent the day. The significant differences of the values (shown above bars) indicate morning anticipation is severely disrupted in *dom* RNAi lines. **C.** Actograms showing the average activities on the last day of LD followed by 5 days in DD. Light represents the day and gray darkness. From left to right: (Left panel) Gal4 control; (middle panel) $dom^{RNAi\#1}$ flies; and (right panel) $dom^{RNAi\#2}$ flies (knockdown of *dom* in all circadian neurons). Depletion of DOM caused arrhythmia in DD.

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INTeractome) scoring [32], we decided to further investigate its role in circadian regulation. Interestingly, prior studies indicated that *dom* mRNA levels in the sLN_vs are clearly enriched about 4.4-fold compared to other neurons [33].

Homozygous *dom* mutants are lethal, therefore we used RNAi to deplete DOM in circadian neurons. *Dicer 2* was co-expressed to enhance RNAi efficiency and has been shown to have no effects on circadian rhythms [34, 35]. When we expressed *dom* dsRNAs using *tim-GAL4*, a circadian tissue-specific driver [36], most of the flies became arrhythmic, and the amplitude of rhythm was significantly reduced (Fig 1A and S1 Table). Only 31% of flies with *dom* downregulation (41% for another independent line *dom* RNAi#2) were rhythmic, and interestingly the circadian period of these lines was about 1.5 hours longer than the control. The reduced rhythmicity and amplitude were also observed when we only targeted the PDF positive LN_vs using *pdf-GAL4* [11] (Fig 1A). However, no reduction on circadian rhythmicity was observed when we targeted all PDF negative circadian cells, using a combination of *tim-GAL4* with the repressor transgene *pdf-GAL80* (Fig 1A and S1 Table). Only a slight period-lengthening of activity rhythm (~0.4hr) was detected. These results indicated that DOM primarily functions in the LN_vs to control circadian behavior, but could function in PDF negative circadian neurons to fine-tune circadian period length.

Because of the high arrhythmia in DD, we also examined the behavioral phenotype of *dom* downregulation in *tim*-expressing circadian neurons under LD. We found that DOM knockdown abolished the morning peak of anticipatory activity in flies (Fig 1B). Importantly, two independent *dom* RNAi lines targeting non-overlapped regions of *dom* exhibited similar phenotypes in both LD and DD (Fig 1C and S1 Fig). Thus, it is unlikely that off-target effect of *dom* RNAi cause these circadian phenotypes. Furthermore, quantitative RT-PCR results showed that *dom* expression were significantly reduced in fly heads of DOM knockdown as compared to controls (S1 Fig). Together, these results suggest that DOM is important for regulation of circadian locomotor behavior.

DOM regulates the abundance of PER and TIM

Depletion of DOM severely decreased the rhythmicity and lengthened circadian period. To understand how DOM affects circadian rhythms, we first measured the oscillations of mRNA abundance of three core pacemaker genes in fly heads with expression of *dom* dsRNAs using *tim-GAL4*. For *clk*, the abundance and oscillation of mRNA under LD and DD were not affected in *dom* RNAi flies (Fig 2A). However, we found that the abundance of *per* and *tim* mRNA was reduced, especially at the time point of peak expression under LD (Fig 2A). Under

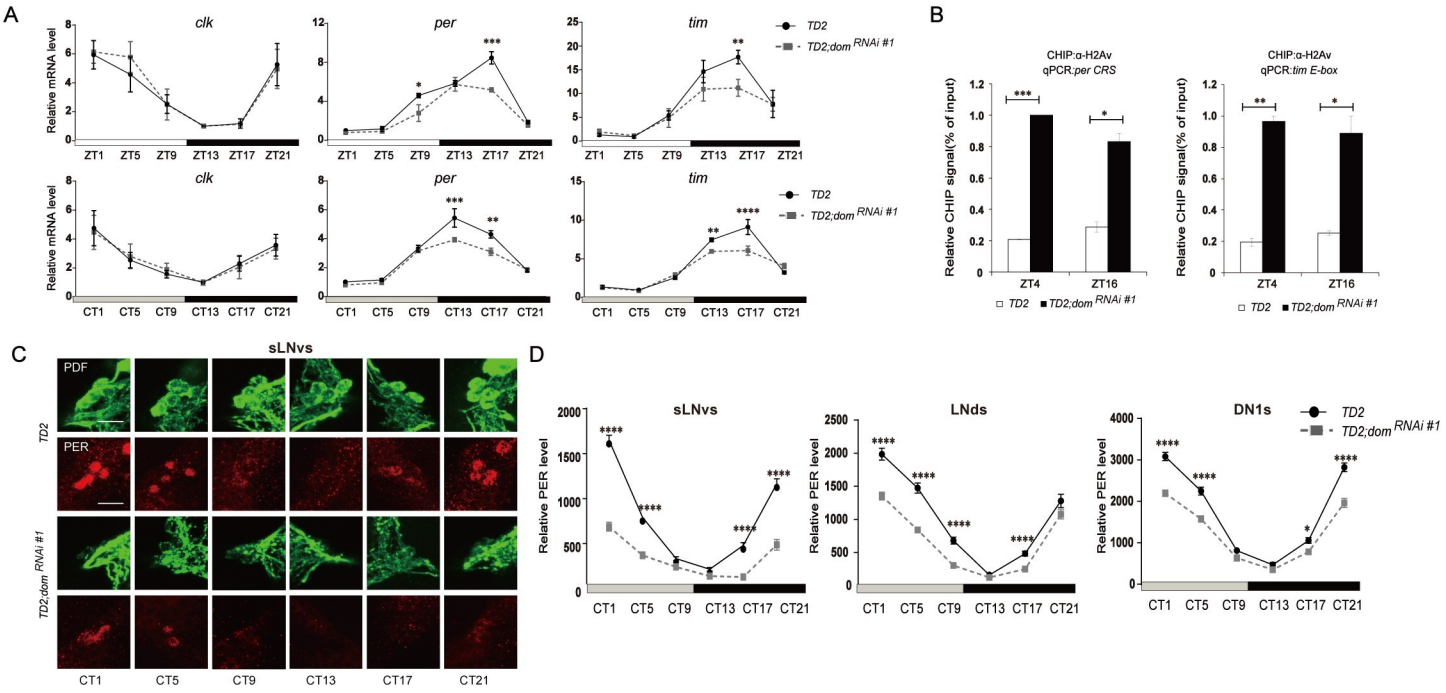

Fig 2. Downregulation of DOM decreases the abundance of PER and TIM. A. Quantitative RT-PCR showing the expression of *clk*, *per* and *tim*. Flies were collected at the indicated time points (ZT = Zeitgeber Time or CT = circadian time). Downregulation of *dom* decreased *per* and *tim* mRNA levels (middle and right panels), while *clk* was normal (left panel). B. ChIP assays detecting H2Av binding more at the *per CRS* and *tim E-box* in flies downregulation of *dom* in *tim*-expressing cells with dicer2 as compared to control TD2 flies. Non-specific DOM binding was detected by amplifying an intergenic region (FBgn0003638) of the *Drosophila* genome and subtracted from the signal from the *per CRS* and *tim E-box 1* signals. Results shown are from at least three biological ChIP replicates, with technical triplicates performed during qPCR for each biological replicate. Error bars represent ± SEM; n.s., non significant,$^{*}P < 0.05$,$^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. **C.** Whole mount immunostaining showing the expression pattern of PER in sLN$_v$s. Red represents PER and green is PDF. Flies were entrained for 4 days in LD and transferred to DD and dissected every 4 h on the fifth day. Downregulation of DOM decreased PER levels at CT1-5 and CT17-21. (Scale bar: 50 um.) **D.** Quantification of the staining in sLN$_v$s, LNds and DN1s. For each genotype, 16–20 fly brains and 60–80 neurons were used for quantification. White and black bars indicate lights-on and lights-off, respectively. Gray and black bars indicate subjective day and subjective night, respectively. Time (h) is indicated as ZT or CT where CT0 is 12 h after lights-OFF of the last LD day. Two independent experiments were done for each genotype/condition with very similar results. Error bars correspond to SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ as determined by the *t*-test.

https://doi.org/10.1371/journal.pgen.1008474.g002

DD, depletion of DOM also decreased the level of *per* and *tim* mRNA, which indicates that the effects are not due to masking effects of light (Fig 2A). We hypothesized that DOM may facilitate CLK-driven rhythmic transcription by regulating the balance between H2Av and phosphor-H2Av via binding to CLK. So we examined the H2Av occupancy at *per* and *tim* promoter by performing chromatin immunoprecipitation (ChIP) in fly heads using the commercialize *Drosophila* H2Av antibodies. We entrained flies under LD and collected head samples at ZT4 and ZT16, close to the trough and peak time of CLK binding [37]. Consistent with our hypothesis, we observed a significant increase in H2Av binding at both promoters when *dom* is knocked down in *tim*-expressing circadian cells after normalized to an intergenic region control (Fig 2B). This data indicate that DOM interacts with CLK to regulate transcription of circadian genes by replacing H2Av at the promoters. However, we did not observe significant difference in binding between ZT4 and ZT16 (Fig 2B), which indicates that the effect of DOM might not be time-dependent.

Next we examined the oscillation of PER protein in major groups of pacemaker neurons with *tim-GAL4* expressing *dom* dsRNA across 24 hours under DD. Consistent with the changes at mRNA level, the level of PER was also significantly reduced in the sLN_vs with DOM depletion (Fig 2C and 2D). In other groups of circadian neurons, such as the LNds and DN1s, decreased abundance of PER was also observed (Figs 2D and S2). Taken together, our results suggest that DOM regulates the H2Av occupancy at promoters of circadian genes and controls the abundance of PER.

DOM-A but not DOM-B is regulated by CLK

There are two major splice variants of *dom*: *dom-A* and *dom-B*, which has distinct functions during *Drosophila* oogenesis [25]. We wondered whether these two isoforms would have different functions in circadian rhythms. We first examined the expression pattern of *dom-A* and *dom-B* in the fly head across different times of the day. Interestingly, *dom-A* exhibited a strong oscillation pattern with a trough around zeitgeber time 9 (ZT9, as ZT0 is light on and ZT12 is light off) and a peak expression near ZT21 (S3A Fig). Oscillation of *dom-A* was also observed under DD. In addition, the oscillation of *dom-A* expression was abolished in the *Clock* null mutant Clk^{out} (S3A Fig) [38]. Together, these data indicate that *dom-A* is controlled by circadian clock. However, we did not observe a clear oscillation for *dom-B* expression (S3B Fig). In order to detect changes at protein level, we took advantage of isoform-specific antibodies to DOM-A or DOM-B [25]. We confirmed the specificity of these antibodies by altering the level of DOM-A or DOM-B using RNAi and overexpression (S3C and S3D Fig). Similar to mRNA level, DOM-A protein abundance was dramatically reduced in Clk^{out}, however, no oscillation of DOM-A was observed in wild type flies (S3E and S3F Fig). This loss of DOM-A oscillation at protein level indicates that DOM-A protein might be quite stable. The levels of DOM-B were not affected in Clk^{out} (S3G and S3H Fig).

DOM-A and DOM-B have distinct roles in circadian regulation

Differential regulations of DOM-A and DOM-B by CLK suggest that they might have different roles in circadian rhythm. We therefore performed isoform-specific downregulation in circadian tissues using small hairpin RNA (shRNA) targeting *dom-A* or *dom-B*. These transgenic fly lines have been previously shown to specifically knockdown these alternative isoforms (S1 Fig) [25]. We first quantified the efficiency of *dom-A* and *dom-B* downregulation by expressing shRNAs in circadian cells of fly heads using quantitative real-time PCR. Consistent with previous report [25], we observed that each shRNA line specifically downregulated *dom-A* or *dom-B* (S1B Fig). Interestingly, when we depleted DOM-A or DOM-B in all circadian clock neurons

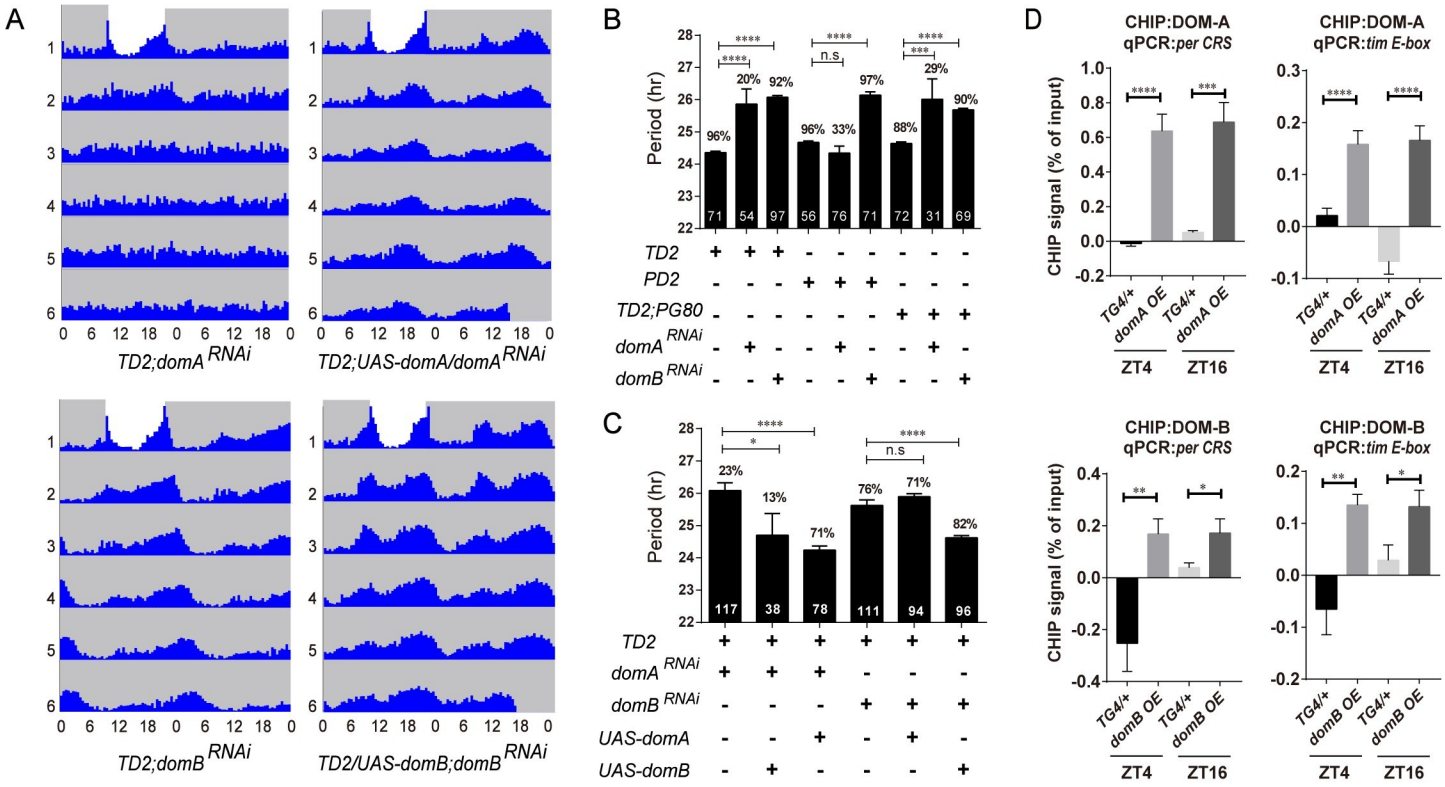

Fig 3. DOM-A and DOM-B have distinct functions in regulating circadian locomotor rhythms. A. Actograms showing the average activities on the last day of LD followed by 5 days in DD. Light represents the day and gray darkness. From top to bottom: $domA^{RNAi}$ (Top left panel); $domA^{RNAi}$;*UAS-domA rescue*(Top right panel); and $domB^{RNAi}$ (Bottom left panel); $domB^{RNAi}$;*UAS-domB rescue*(Bottom right panel). Depletion of *domA* caused arrhythmia in DD, while knocking down *domB* lengthened circadian period. $domA^{RNAi}$ and $domB^{RNAi}$ phenotypes can be rescued by restoring *domA* and *domB* expressing in all circadian neurons respectively. **B.** Free-running period and percentage of rhythmicity of *domA* and *domB* depleted flies. The percentages of rhythmic flies are shown above the error bar. The numbers of tested flies are shown in each column. **C.** Free-running period and percentage of rhythmicity of flies with restoring DOM-A or DOM-B in *domA* or *domB* depletion. **D.** ChIP assays detecting DOM-A and DOM-B binding more at the *per CRS* and *tim E-box* in flies expressing *domA* (BL64261) and *domB* (BL64263) in tim-expressing cells as compared to control TG4 flies. Quantification was done same as Fig 2B. Error bars represent ± SEM; n.s., non significant,$^{*}P < 0.05$,**p < 0.01, ***p < 0.001, $^{****}P < 0.0001$; one-way ANOVA.

https://doi.org/10.1371/journal.pgen.1008474.g003

by *tim-GAL4*, we observed two distinct circadian behavioral phenotypes. Most of the flies expressing *dom-A* shRNA lost locomotor rhythms, except for ~20% that were rhythmic and showed a longer period as compared to the control (Fig 3A and 3B; S1 Table). As with depletion of all *dom* isoforms, DOM-A specific knockdown also blunted the morning activity peak under LD (S4 Fig). However, for DOM-B knockdown, flies exhibited a lengthened period of ~1.5 hrs in DD (Fig 3A and 3B), but no effect on amplitude of rhythms or morning anticipation was observed under LD (Fig 3B and S4 Fig). When we expressed *dom-A* or *dom-B* shRNA only in LN_vs using *pdf-GAL4*, or in PDF negative circadian neurons, we found similar effects on rhythmicity or period-lengthening (Fig 3B). These results suggested that DOM-A and DOM-B are required in both PDF positive and negative circadian neurons for behavior. It is unlikely that the different circadian phenotype is due to greater efficiency of *dom-A* over *dom-B* shRNA (S1B Fig). Actually a previous study has also validated the knockdown efficiency and found that *dom-B* shRNA is in fact slightly stronger than *dom-A* in the fly nervous system [25]. However, we cannot fully exclude the possibility that DOM-A protein is more stable than DOM-B and cause this difference of circadian phenotypes.

Our isoform-specific knockdown indicated that DOM-A and DOM-B have distinct functions in circadian rhythms. Although the shRNA lines have been shown here and in a previous

study [25] to specifically knockdown the *dom-A* or *dom-B* isoforms, off-target effects could still contribute to the observed phenotypes. Thus, we performed rescue experiments with UAS transgenic flies expressing either *dom-A* or *dom-B* cDNA. Circadian behavior defects were rescued when we overexpressed corresponding UAS lines in the DOM-A or DOM-B knockdown (Fig 3A and 3C), which indicated that the isoform specific phenotype we observed was not due to off-target effects. Remarkably, with overexpression of *dom-A*, we were not able to rescue the long period phenotype of DOM-B knockdown (Fig 3C, and S1 Table). Similarly, overexpression of *dom-B* could not rescue the arrhythmic phenotype of DOM-A knockdown, although we did notice that the period-lengthening effect was partially rescued in the remaining 13% of rhythmic flies (Fig 3C).

Since we observed DOM regulates the occupancy of H2Av at *per* and *tim* promoters, we therefore examined whether DOM-A and DOM-B might be bound to the promoters of *per* and *tim*. We used the DOM-A- and DOM-B-specific antibodies to perform ChIP in fly heads with overexpression of *dom-A* or *dom-B* at two time points: ZT4 and ZT16. We observed significant enrichments of DOM-A and DOM-B binding on both *per* and *tim* promoters, compared to an intergenic region control (Fig 3D). Similar with H2Av ChIP, we did not observe significant difference in binding between ZT4 and ZT16 (Fig 3D), which indicates that the binding of DOM-A and DOM-B might not be time-dependent.

DOM-A, but not DOM-B, is required for the maintenance of sLN_vs and PDF abundance

The blunted morning activity peak and arrhythmia in DOM and DOM-A knockdown flies suggest that there might be defects in the sLN_vs or in PDF signaling. There are three major projections of PDF positive LN_vs. The lLN_vs send projections to the optic lobes and the contralateral brain hemisphere, while the sLN_vs send projections to the DN1s and DN2s region [39]. Based on whole mount immunohistochemistry in fly brains, we did not observe obvious defects in the brain structure or projections of the lLN_vs, however, PDF levels in the dorsal projections of the sLN_vs were barely detectable in DOM and DOM-A knockdown flies (Fig 4A). Absence of the dorsal projection or low PDF expression in the sLN_vs could lead to decrease of PDF in the dorsal projection [20, 40]. Our data are consistent with both possibilities. Using *pdf-GAL4* and a membrane tethered GFP (CD8-GFP) to label axons of sLN_vs, we observed that the dorsal projections of sLN_vs were clearly shortened in DOM and DOM-A (Fig 4B–4D). Interestingly, the sLN_vs dorsal projections were normal in DOM-B downregulation, which is consistent with the behavior results. Furthermore, close observation of the PDF positive sLN_vs revealed that both the number of sLN_vs and PDF levels were reduced (Fig 5A and 5B). For each hemisphere, the average number of PDF positive sLN_vs in DOM and DOM-A knockdown flies was approximately 2, compared to 4 in the control flies or DOM-B knockdown (Fig 5A and 5B). However, with DOM-B knockdown, we did not observe any obvious loss of sLN_vs or reduction in PDF levels, which indicates that this process is specifically controlled by DOM-A (Fig 5A and 5B). Consistent with the ChIP results, the levels of PER and TIM were found to be significantly decreased in the sLN_vs with knockdown of DOM-A or DOM-B (Fig 5A and 5B). This may also explain why depletion of DOM-A or DOM-B had an effect on period-lengthening.

Next we examined which step of regulation causes the reduction of PDF levels in DOM and DOM-A knockdown. DOM regulates gene expression at the transcription level [24]. We therefore examined the expression of *pdf* using a transcriptional reporter line, *pdf*Tomato [21]. Consistent with PDF staining, we observed significant reduction of *pdf* transcription in sLN_vs of both DOM and DOM-A knockdown (Fig 5C and 5D). Interestingly, the decrease in neuron

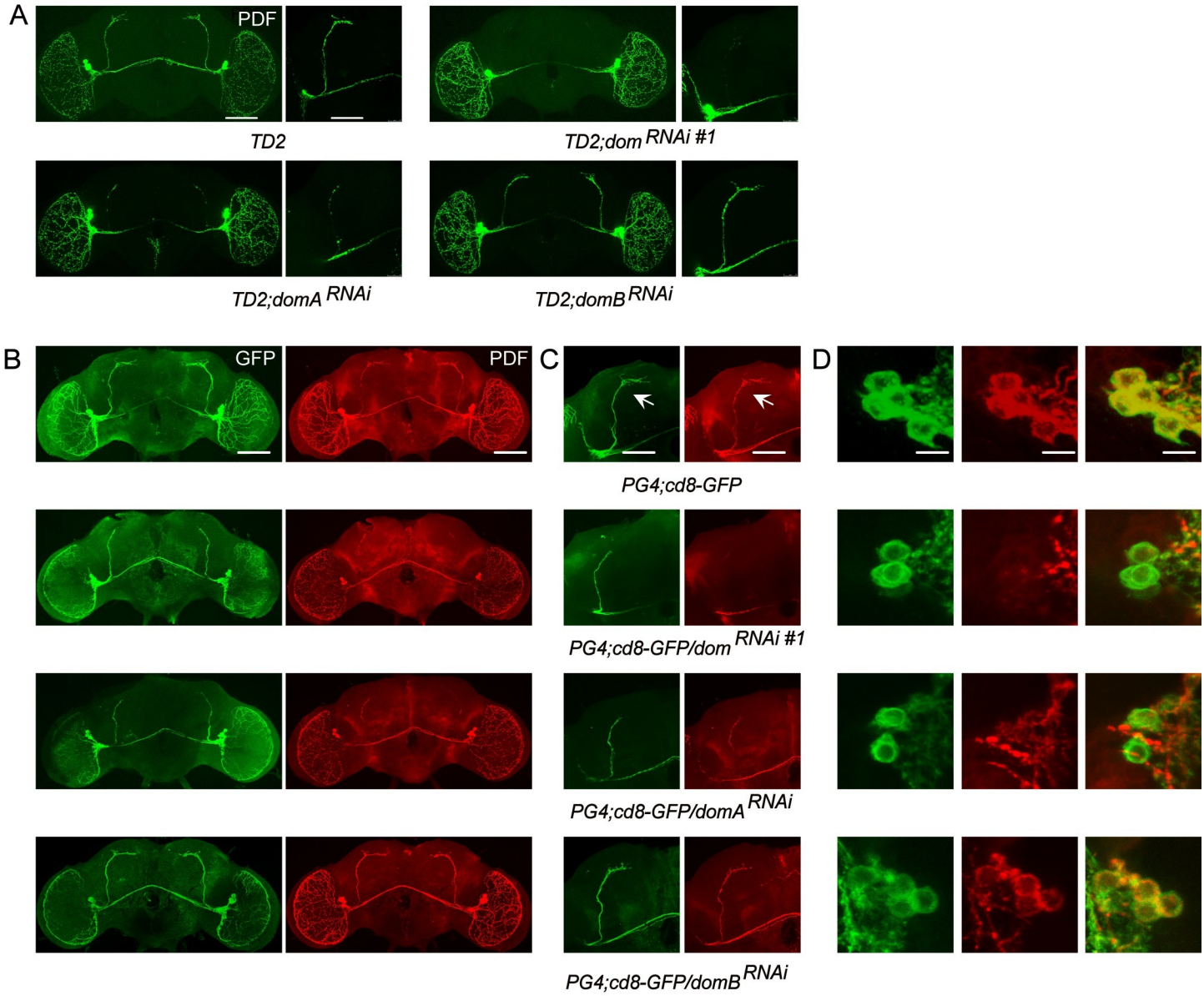

Fig 4. Eliminations of dorsal projections in DOM and DOM-A downregulation were due to reduced number of sLNvs and decrease of PDF. A. Representative confocal images showing PDF expression in whole brain and dorsal axonal projection of sLN_vs. Brains were dissected at ZT1 for anti-PDF (green) and anti-PER antibodies (red). Comparing to the control (Top left panel), dorsal projection of sLNvs was disrupted in *dom* (Top right panel) and *domA* downregulation (Bottom left panel) no effects were observed in the presence of *domB* RNAi (Bottom right panel) (Scale bar: left, 500 um; right, 100 um). **B.** Associated genotypes adults brains collected at ZT1 were immunostained with GFP (green) and PDF (red) antibodies and imaged (see Materials and Methods) (Scale bar: 500 um). **C.** Representative confocal images of dorsal axonal projection of sLN_vs (Scale bar: 100 um). **D.** Representative images of sLN_vs of associated lines. Merged GFP+PDF images were shown as yellow (Scale bar: 50 um).

https://doi.org/10.1371/journal.pgen.1008474.g004

number and reduction of PDF level were unique to sLN_vs, which were not detected in the lLN_vs (Fig 5C and 5D).

In summary, consistent with differences in circadian behavior, DOM-A and DOM-B knockdown also exhibit differences at the molecular level. Knockdown of DOM-A causes loss of sLN_vs and decrease in sLN_v PDF levels, which is not observed in DOM-B downregulation.

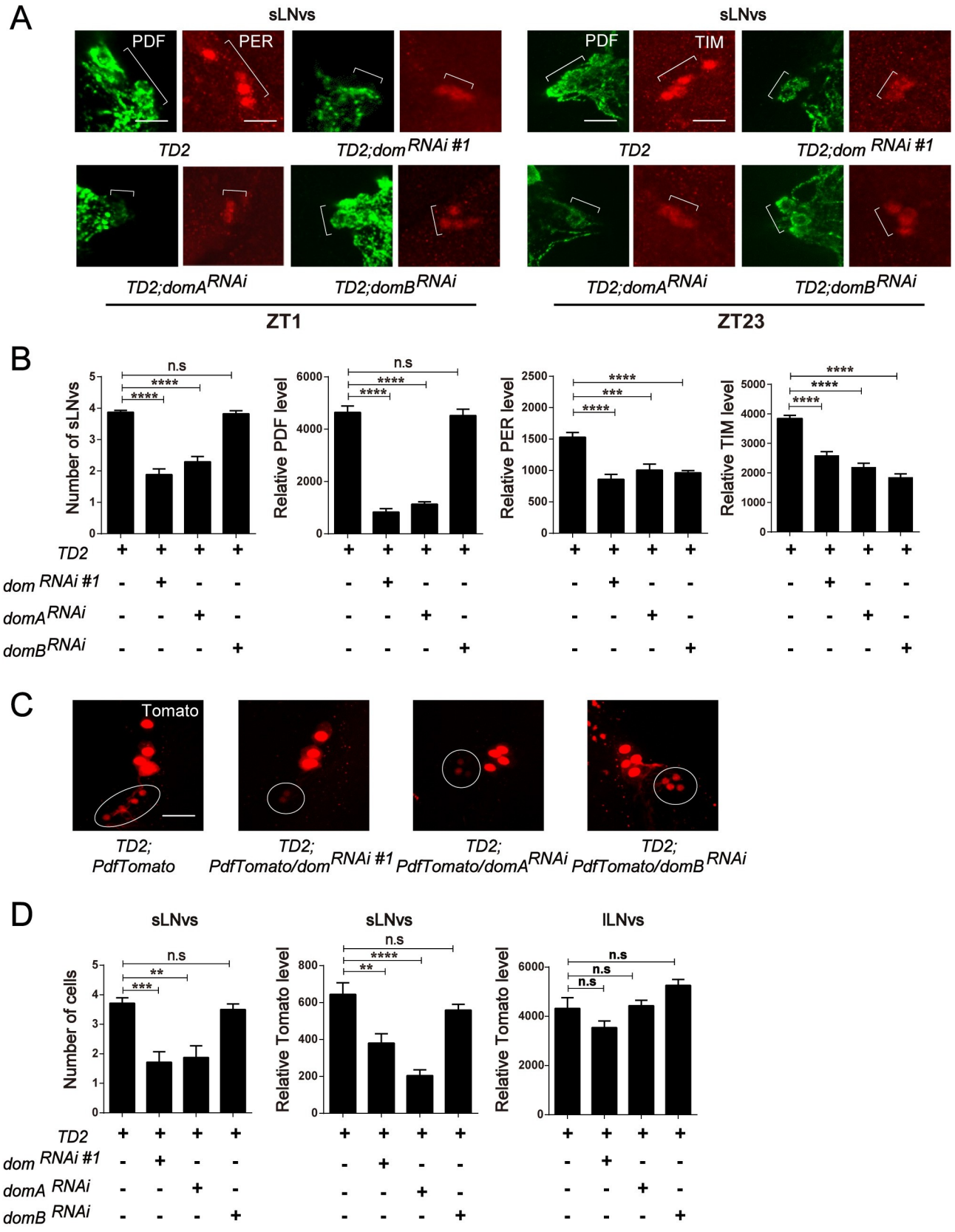

Fig 5. Depletion of DOM-A, but not DOM-B affects s-LN$_v$s maintenance and PDF accumulation. A. Representative images of sLN$_v$s of associated lines. Brains were dissected at ZT1 for anti-PDF (green) and anti-PER antibodies (red) or dissected at ZT23 for anti-PDF (green) and anti-TIM

antibodies (red). Cell number of sLN_vs (marked as square brackets), PDF, PER and TIM levels in sLN_vs were decreased in *dom* and *domA* downregulation flies brains (Top right and bottom left panels), while only affected PER and TIM levels in *domB* downregulation flies (Bottom right panel). (Scale bar: 50 um). **B.** Quantification of sLN_vs numbers in each brain hemisphere, as well as PDF, PER and TIM levels in sLN_vs. n = 32 hemispheres was used for quantification. **C.** *Pdf* transcriptional level in lLN_vs and sLN_vs using TOMATO fluorescence signal. *Pdf* transcriptional levels in sLNvs (marked as circle) were decreased in *dom* and *domA* downregulation fly brains (middle two panels), while sLNv numbers and *pdf* transcriptional levels in *domB* downregulation flies were normal (right panel) (Scale bar: 100 um.). **D.** Quantification of sLN_vs numbers and TOMATO fluorescence signal in sLN_vs and lLN_vs. n = 24 hemispheres were used. Error bars correspond to SEM. $^{**}p < 0.01$,$^{***}p < 0.001$,$^{****}p < 0.0001$ as determined by t test.

https://doi.org/10.1371/journal.pgen.1008474.g005

DOM is required during development and adulthood for circadian rhythms

Decrease of sLN_vs numbers and dorsal projections in adult flies might be due to abnormal development or maintenance defects. Therefore, we performed brain dissections at larval stage and pupal stage. The number of sLN_vs precursor in the DOM knockdown condition was unchanged compared to the control at 3rd instar larvae (Fig 6A and 6B), which suggests that DOM does not affect sLN_vs development. However, same as adulthood, even at early pupal stage (3 days after pupation), there were only 1–2 sLN_vs detected in *dom* downregulation (Fig 6C and 6D). These data indicate that DOM is required for the maintenance but not for the development of sLN_vs. DOM probably affects sLN_vs during larval-pupal metamorphosis. Consistent with adult flies, both PDF and PER levels of *dom* downregulation were reduced in sLN_vs compared to the control in larvae or pupae (Fig 6B and 6D).

The decrease of axonal projections and number of sLN_vs we observed in DOM knockdown flies (Figs 4 and 5) suggested that DOM might play a role during development to properly regulate circadian rhythms. Thus, we used *tim-GAL4* and GAL80^{ts} TARGET system to temporally express *dom* dsRNA during development or after eclosion [41]. GAL80 is active at 18˚C thus blocks GAL4 function and prevents dsRNA production, while 30˚C inactivates GAL80 and allows expression of dsRNA to downregulate DOM. When we grew flies at 18˚C and tested circadian behavior at 30˚C, we observed strong rhythmicity but around 1–2 hr period-lengthening with adult-specific DOM or DOM-A knockdown (Fig 6E and S1 Table). However, knockdown DOM-A only during development led to high arrhythmic activity (Fig 6E and S1 Table). Thus, developmental expression of *dom* or *dom-A* dsRNA appears to affect the rhythmicity, while adult specific depletion appears to be sufficient for period-lengthening. Furthermore, knockdown of DOM-B only in adulthood caused a ~1 hr increase in period length, suggesting that unlike DOM-A, the DOM-B splice form is mainly necessary for the regulation of circadian clocks in adult stage (Fig 6E and S1 Table). We observed a slight but significant lengthening of period (~0.6 hr, S1 Table), when we specifically depleted DOM-B in circadian neurons during development, which indicates that DOM-B might also play a role during development.

To exclude anatomical defects in the circadian circuitry, we grew flies at 18˚C and entrain them under LD for 5 days at 30˚C, then performed brain dissections at ZT1. As expected, the dorsal projections and number of sLN_vs in the flies with adult-specific DOM downregulation were normal, and PDF abundance was not affected (Fig 6F and 6G). These data further suggest that the arrhythmia we observed in DOM knockdown was mainly due to the function of DOM during development. Indeed, the abundance of PER in sLN_vs was still reduced, which could explain the lengthened period phenotype (Fig 6G). Since we observed DOM binding to the *per* and *tim* promoters, and since DOM is required at adulthood for the period-lengthening, we checked whether adulthood specific downregulation of DOM affects *per* and *tim* transcription by using quantitative RT-PCR. The pattern of *per* and *tim* oscillation was still present in adult-specific DOM knockdown, however the abundance of transcripts is dramatically reduced, especially for the early night time points of *per* (S5 Fig), which is consistent with the PER protein level (Fig 6F and 6G).

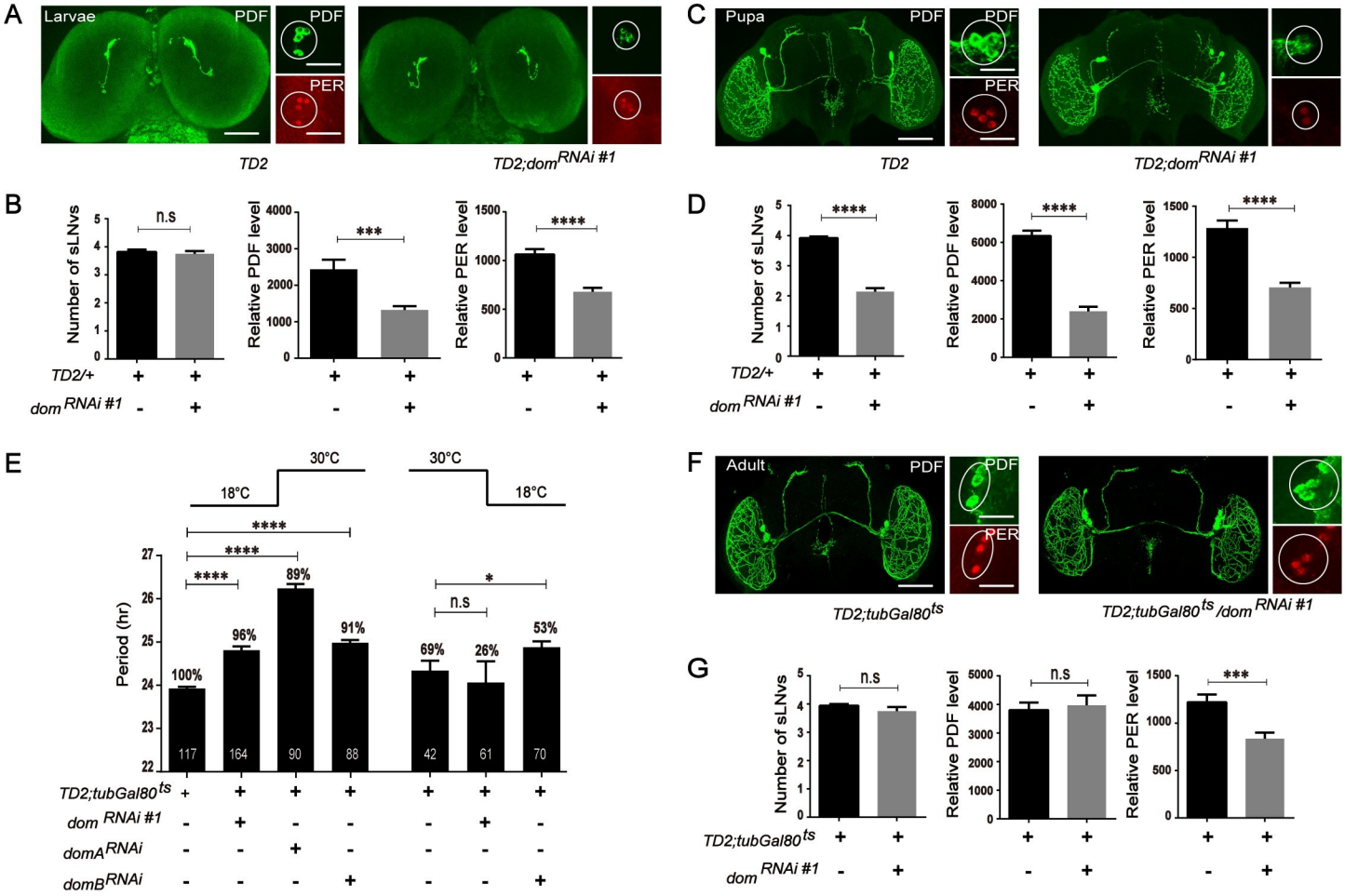

Fig 6. DOM-A is required during development for circadian rhythmicity and adulthood for regulation of period length, while DOM-B is only required in adulthood. A. Representative larva brain confocal images. L3 stage larvae collected at ZT1 were immunostained with PDF and PER antibodies. Silence the expression of *dom* in larvae stage affected PDF and PER levels in sLNvs (marked as circle), while number of sLNvs were not affected (Scale bar: left, 200 um; right, 50 um.). **B.** Quantification of larva sLN$_v$s, as well as PDF and PER levels. For each genotype, ~22 flies brains and ~85 neurons were used for quantification. **C.** Representative pupal brain confocal images. Flies pupa (3 days after pupation) were collected at ZT1 were immunostained with PDF and PER antibodies. Silence the expression of *dom* in pupal stage affected number of sLNvs, PDF and PER levels in sLNvs (marked as circle) (Scale bar: left, 500 um; right, 50 um). **D.** Quantification of pupal sLN$_v$s, as well as PDF and PER levels. For each genotype, ~25 flies brains and ~100 neurons were used for quantification. **E.** Free-running period and percentage of rhythmicity of DOM, DOM-A and DOM-B depleted flies in adulthood (left part) or during development (right). Stage specific silencing the was done by using the conditional *tim-Gal4;tub-Gal80^{ts}* driver system. **F.** Representative confocal images of fly brain showing projections of PDF positive LN$_v$s. Flies were grown at 18˚C until eclosion, and adult flies were entrained for 5 days in LD 30˚C. Brains were dissected at ZT1 for anti-PDF (green) and anti-PER antibodies (red) (Scale bar: left, 500 um; right, 50 um). **G.** Quantification of the numbers of sLNvs and PDF, PER levels in sLN$_v$s of associated lines. Error bars correspond to SEM. $^{*}P < 0.05$,$^{***}p < 0.001$, $^{****}p < 0.0001$ as determined by the *t*-test.

https://doi.org/10.1371/journal.pgen.1008474.g006

Taken together, these data indicate that DOM/DOM-A is required during development for the rhythmic activity, while both DOM-A and DOM-B are necessary for period determination post development.

Activation of PDFR signaling in circadian neurons rescues the behavioral phenotype of DOM depletion

Since we observed low PDF levels in the sLN$_v$s with DOM knockdown, we wondered whether the decrease of PDF signaling was the cause of arrhythmia. To test this hypothesis, we determined whether hyperactivation of PDFR could rescue the phenotypes that accompany reduction in DOM expression. To increase PDFR signaling, we expressed a membrane-tethered

PDF (t-PDF) in circadian cells with *tim-GAL4*, which has been shown to mimic high PDF levels [42]. A scrambled peptide sequence (t-PDF SCR) with similar length as PDF was used as a negative control. Strikingly, co-expression of t-PDF using *tim-GAL4* restored the rhythmicity to 89% for *dom* RNAi (71% for another independent *dom* RNAi line)(Fig 7A and 7B), whereas the t-PDF SCR control was unable to restore rhythmicity (Fig 7A and 7B). Not only the rhythmicity, but also the period of DOM knockdown was rescued with co-expression of t-PDF (Fig 7B). To exclude the possibility that the behavior rescue in t-PDF was due to ceased expression of *dom* dsRNA, we measured the *dom* transcripts in fly heads. Co-expression of t-PDF with *tim-GAL4* downregulated *dom* to the similar level as *dom* RNAi control (S6A Fig). Since the circadian period was also restored in dom knockdown with expression of t-PDF, we then stained fly brains with PDF and pacemaker proteins PER and TIM (Fig 7C and S6B Fig). With expression of t-PDF in DOM knockdown flies, we made two interesting observations. First, PDF level as well as the dorsal projection of sLN_vs were restored, while the number of sLN_vs was still lower (Fig 7C and 7D). Second, the abundance of PER and TIM in sLN_vs was also restored (Fig 7C and 7D and S6B and S6C Fig). Together, our data showed that activation of PDFR signaling in circadian neurons rescues the arrhythmic and period-lengthening phenotype of *dom* knockdown.

Discussion

Here, we identify that two alternatively spliced variants of DOM play distinct roles in circadian rhythms. Both DOM-A and DOM-B determine the circadian period of locomotor rhythms by regulating core pacemaker protein PER/TIM abundance. However, DOM-A plays specific roles for maintenance of sLN_vs and PDF abundance during development thus controls morning anticipatory activity and circadian rhythmicity.

The abolished morning anticipatory activity in light dark cycle and low rhythmicity under constant darkness in DOM/DOM-A knockdown flies is reminiscent of the phenotypes seen in *pdf* mutant (Fig 1 and Fig 3) [43]. Based on these observations, we hypothesized that PDF signaling is disrupted with *dom* or *dom-A* RNAi. Indeed, we found that PDF abundance was decreased both in the soma and dorsal axonal projections of sLN_vs (Fig 4). Furthermore, by restricting RNAi to adulthood, we restored the PDF expression and projection, which also restored the rhythms (Fig 6). These results suggest that the arrhythmic phenotype is due to the decrease of PDF levels in *dom* or *dom-A* downregulation.

Despite the importance of sLN_vs in circadian rhythms, the mechanism underlying sLN_vs maintenance is still unclear. Here we find that DOM-A plays important roles in the maintenance of sLN_vs. PDF positive sLN_vs and dorsal projections are formed in first instar larvae [10]. During development, both DOM-A and DOM-B start to be expressed in embryos [23]. Our data suggest that DOM-A does not affect sLN_vs development in larvae, but rather regulates sLN_vs maintenance at later stages (Fig 6). Knockdown of DOM-A may trigger apoptosis or necrosis pathways and cause programmed cell death in sLN_vs. In fact, chromatin-modifying pathway has been shown to regulate neuronal necrosis in flies [44]. In the future, it will be interesting to examine whether DOM-A is involved these programmed cell death pathways and blocking necrosis or apoptosis pathway can restore the sLN_vs in DOM downregulation.

Here we found that depletion of DOM-A seems to specifically affect the maintenance of PDF positive sLN_vs and PDF accumulation in sLN_vs, while the PDF positive lLN_vs is not affected (Fig 5). This suggests that as master pacemaker neurons, sLN_vs may have some unique regulatory program for maintenance and gene expression, which requires DOM-A. Interestingly, a phosphatase LAR has recently been found to specifically regulate PDF expression in the dorsal projection of sLN_vs during development [40]. The pacemaker protein VRI also

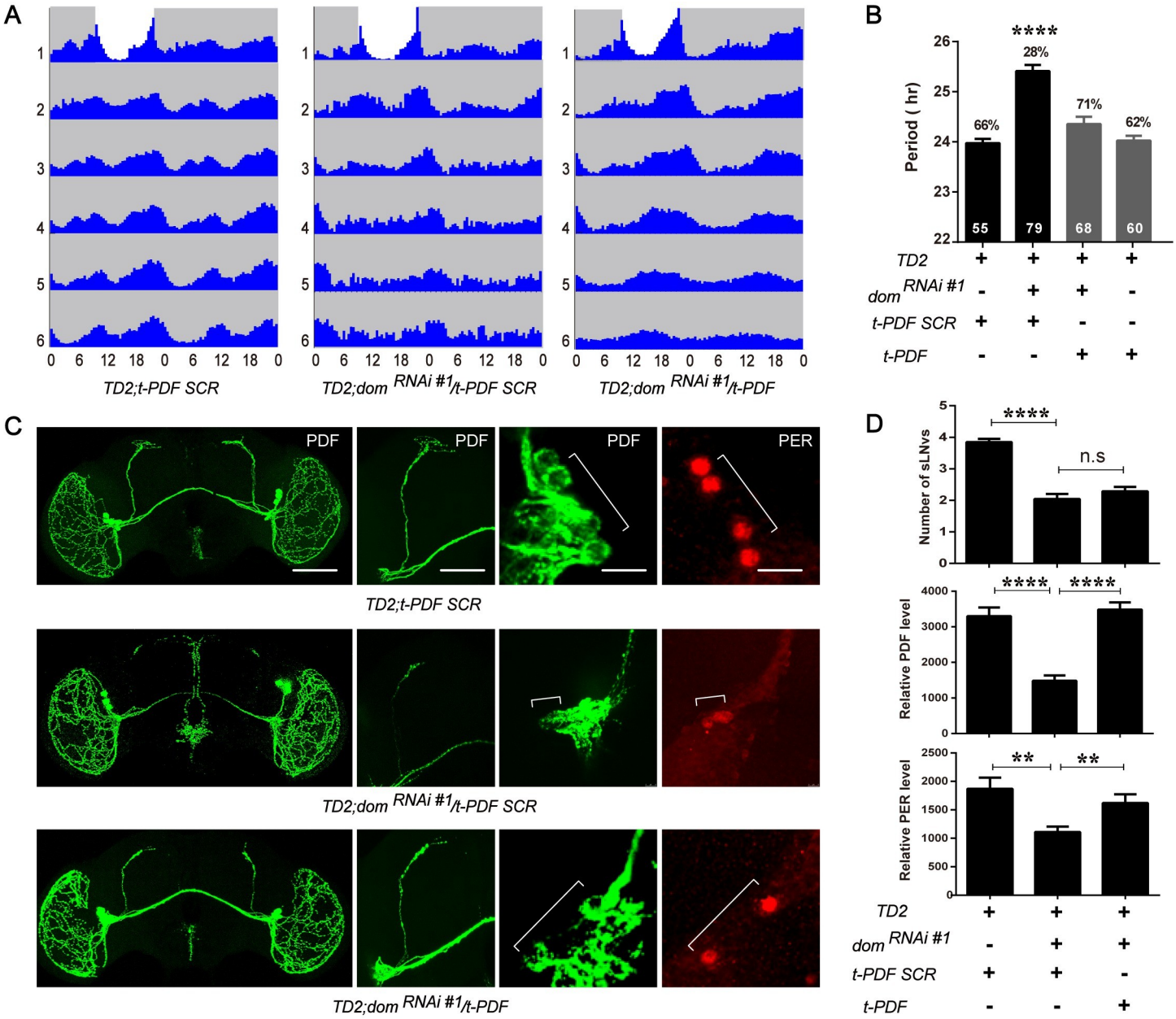

Fig 7. Constitutive activation of PDFR signaling rescued the circadian behavior phenotype of DOM downregulation. A. Actograms showing the average activities on the last day of LD and during 5 days in DD. Light represents the day and gray darkness. From left to right: (Left panel) flies expressing the membrane-tethered scrambled PDF; (middle panel) $dom^{RNAi\#1}$ flies expressing a membrane-tethered scrambled PDF (negative control); and (right panel) $dom^{RNAi\#1}$ flies expressing the membrane-tethered PDF. Arrhythmia and long period of *dom* downregulation are rescued with tethered PDF. **B.** Free-running period of *dom* RNAi flies expressing the membrane-tethered PDF. **C.** Representative confocal images of brains of *dom* RNAi flies expressing the membrane-tethered PDF or scrambled PDF. Flies were entrained for 4 days in LD 25˚C, and brains were dissected at ZT1 for anti-PDF antibody (green) and anti-PER antibody (red). From top to bottom: (Top panel) fly brain expressing the membrane-tethered scrambled PDF; (middle panel) $dom^{RNAi\#1}$ flies expressing a membrane-tethered scrambled PDF; and (bottom panel) $dom^{RNAi\#1}$ flies expressing the membrane-tethered PDF. Confocal images are whole brain, dorsal projection and soma of sLN_vs from left to the right (Scale bar: whole brain, 500 um; projection, 100 um; sLNvs, 50 um). **D.** Quantification of the number and relative PDF and PER levels of sLNvs. For each genotype, totally, 20–25 flies brains and 50–80 neurons were used for quantification of the staining. Error bars correspond to SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$ as determined by *t*-test.

https://doi.org/10.1371/journal.pgen.1008474.g007

played important roles in controlling PDF abundance and dorsal arborization rhythms of sLN_vs [22]. Future efforts to identify specific regulatory mechanisms in sLN_vs will help us understand the maintenance and function of circadian neuronal network.

Why does DOM-A, but not DOM-B, regulate sLN_v maintenance and *pdf* transcription? One possibility might be that DOM-A and DOM-B are associated with different protein complexes. Compared to DOM-B, DOM-A has two unique domains in the C-terminus: a SANT domain and a poly-Q domain. Both of these two domains are known to mediate protein interactions. Interestingly, only DOM-A is identified to bind the Tip60 complex when Tip60 was purified by a tagged protein subunit from *Drosophila* S2 cells [24]. A previous study finds that depletion of Tip60 or overexpression of a histone acetyltransferase-defective Tip60 decreases axonal growth of the sLN_vs in the fly model of Alzheimer's disease [45]. These results suggest that DOM-A might interact with Tip60 to control the axonal projection and PDF expression of sLN_vs.

Here we found that activation of PDFR signaling by t-PDF in *tim-GAL4* expressing neurons not only restored the circadian rhythmicity but also the length of period and PER abundance in DOM knockdown (Fig 7). How does PDFR signaling affect PER? PDF is an important neuropeptide as both being an circadian output factor and adjusting the molecular clock [16]. After activation, PDFR signaling increases cAMP-PKA pathway and stabilized PER in PDF positive neurons [17]. This may explain why we saw a restoration of PER with co-expression of t-PDF and *dom* dsRNA. In addition to PER, we also observed a restoration of TIM abundance. Previous results on the effect of PDF signaling on TIM in non-PDF circadian cells are controversial [18, 46]. The exact mechanism of how TIM level is rescued by t-PDF here is still unclear.

Lastly, how does DOM control circadian period? This is probably through regulation the balance between H2Av and phosphor-H2Av at the promoter of *per* and *tim* via binding to CLK. Interestingly, compared to the decrease of *per* and *tim* mRNA in DOM downregulation, the master circadian transcription factor *clk* mRNA is not affected. Indeed, we found that from *Drosophila* S2 cell affinity pull-down, CLK interacts with DOM complex (Table 1). Furthermore, DOM-A and DOM-B binding is enriched in the E-box region of *per* and *tim* promoters. Even though no binding differences were observed in DOM-A and DOM-B at ZT4 and ZT16 (Fig 3), it is possible that other subunits in the DOM complex bind rhythmically, leading to rhythmic activity of the complex. Given that the conserved functions of DOM and human SRCAP/p400 in Notch signaling and histone variant exchange, it is possible that similar mechanisms are leveraged to control circadian rhythms in mammals.

Materials and methods

Fly stocks

All the flies were raised on cornmeal/agar medium at 25˚C under a LD cycle. The following strains were used: *yw*,w^{1118}, *yw; tim-GAL4/CyO* [36], *yw;Pdf-GAL4/CyO* [11]; *yw;tim-GAL4/CyO;Pdf-GAL80/TM6B* [47], *tublin-GAL80*^{ts}, *UAS-cd8-GFP*, *clk*^{out}. The following stocks were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/): *UAS-dom*$^{RNAi\#1}$ (BL31054), *UAS-dom*$^{RNAi\#2}$ (BL38941), *UAS-domA* (BL64261) and *UAS-domB* (BL64263). *DomA*^{RNAi} *(sh-domA)* and *domB*^{RNAi} *(sh-domB)* fly lines were generous gifts from Dr. Peter B. Becker. *PdfTomato* line was generated by Dr. Sebastian Kadener.

Behavioral experiments and analysis

For behavioral experiments, adult male flies (2–4 days old) were used for testing locomotor activity rhythms. Flies were entrained for 4 full days LD cycle at 25˚C, using about 500 lux light intensities, and then released into DD at 25˚C for at least 6 days. Locomotor activity was measured with TriKinetics Activity Monitors in I36-LL Percival Incubators. Locomotor activity was averaged over the 4 days entrainment for LD and 6 days for DD. Analyses of period

and power were carried out using FaasX software as previously described [48]. Actograms were generated using a signal-processing toolbox implemented in MATLAB (MathWorks) [49]. For $GAL80^{ts}$ experiments, flies were raised at 18˚C and tested at 30˚C. They were entrained for 5 days and then released in DD for at least 6 days. Morning anticipation was calculated by the ratio of activity counts between 2 hrs before light on and 6 hrs before light on. We first measured the single fly activity counts obtained in twelve 30-min bins between Zeitgeber Time (ZT) 17.5 and ZT24 (6 hr before lights on) and six 30-min bins between ZT22.5 and ZT24 (2 hrs before lights on). The first value is divided by the second to obtain the morning activity index. Morning anticipations of individual flies were then averaged and plotted on the graphs.

Whole-Mount immunohistochemistry

Whole-mount immunohistochemistry for fly brains were done as previously described [50]. Adult fly (3–6 days old) or L3 stage larval brains were dissected in chilled PBT (PBS with 0.1% Triton X-100) at the indicated time points and fixed in 4% formaldehyde diluted in PBS for 30 min at room temperature. For pupal brains dissection, *Drosophila* were fully developed and hatched 7 days after pupation in the same cross, pupa were collected 3 days after pupation using a small wet paintbrush and transfer to a dissection dish. After 2–3 times PBS wash, the pupal brains were dissected and fixed as previously described method. The brains were rinsed and washed with PBT three times (10 min each). Then, brains were incubated with 10% normal Goat serum diluted in PBT to block for 60 min at room temperature and incubated with primary antibodies at 4˚C overnight. For PER, TIM and CLK staining, we used 1:1,500 rabbit anti-PER, 1:2,000 rat anti-TIM (gift from Dr. Rosbash) and guinea pig 1:2,500 anti-CLK (gift from Dr. Hardin), respectively. We used a 1:200 dilutions for mouse anti-PDF and 1:200 for rabbit anti-GFP (DSHB). After six washes with PBT (20 min each), brains were incubated with relative secondary antibody at 4˚C overnight, followed by another six washes with PBT. For *PdfTomato* staining, flies were directly dissected in chilled PBT at the indicated time points and moutained in the medium (VECTOR). All samples were imaged on a Leica Confocal SP8 system, with laser settings kept constant within each experiment. 10 to 12 fly brains for each genotype were dissected for imaging. Representative images are shown. Image-J software (National Institutes of Health [NIH]) was used for PER, PDF, TIM and CLK quantification in 15–30 sLNvs from at least seven brains. For quantification, signal intensity in each sLNv and average signals in three neighboring non-circadian neurons were measured, and the ratio between signals in sLNvs and non-circadian neurons was calculated.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed based on published protocols [31]. Flies entrained in 12 hr light:12 hr dark (LD) conditions at 25˚C for four days were collected at two time-points (ZT4 and ZT16) on the fifth day. Briefly, chromatin was isolated from 500 μl of fly heads homogenized with a dounce homogenizer (Kimble Chase) for 20 strokes using the loose "A" pestle. Homogenate was sieved by a 70 μm cell strainer (Falcon) then centrifuged to remove cell debris. Pellets were cross-linked using formaldehyde. Samples were sonicated using a focused-ultrasonicator (Covaris M220) on setting for 400–500 bp cDNA and then centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected in two 130 μl aliquots for IP and 26 μl was collected for input and frozen at -80C for analysis. Sonicated chromatin was roughly 500 bp in length (<1000 bp). For each IP, 30 μl of a Protein G Dynabead slurry (Life Technologies) was washed then incubated along with the appropriate antibodies for 4 hours at 4˚C with rotation. Amount of antibodies used for ChIP is as follows: anti-H2Av (10 ug/ml,

rabbit, Active Motif), anti-DOM-A (20 μg/ml, rabbit, GenScript) which was generated in our lab (antigen protein sequence designed according to 2008–2349 aa of DOM-A), anti-rabbit-IgG (20 μg/ml, Life Technologies), anti-DOM-B (10 μg/ml, mouse, from Dr. Peter B. Becker), anti-mouse-IgG (10 μg/ml, Life Technologies). Following incubation, beads were collected and incubated with chromatin overnight at 4˚C with rotation. DNA was eluted using the Qiagen PCR purification kit and subjected to qPCR. At least three technical replicates of qPCR were performed for each biological ChIP replicate and three biological replicates were performed for H2Av, DOM-A and DOM-B assays. Background binding to a nonspecific antibody (anti-IgG; Life Technologies) bound to Dynabeads was subtracted from input samples and results are presented as the percentage of the input samples. For each assay, at least three biological replicates were performed. The specific primers used for qPCR are described in S2 Table. The technical qPCR triplicates were averaged for each biological replicate as no significant differences were found between the technical replicates, and the error bars represent SEM calculated from variance between biological replicates. Two-tailed t-tests were used to determine statistical differences between control and experimental treatment at each ZT.

Real time quantitative reverse transcription PCR

Flies were collected at the indicated time points and isolated heads were stored at −80˚C. Total RNA was extracted from 25–30 heads with TRIzol based on the manufactures protocol (Life Technologies, USA). A 2-μg quantity of RNA was reverse-transcribed with reverse transcription reagents (Invitrogen). For real-time PCR (qRT-PCR) of *per*, *tim*, *clk*, *dom*, *domA*, *domB* and *actin*, we used a qPCR detection kit (SYBR Select Master Mix For CFX) (Life Technologies, USA). The specific primers used for qPCR are described in S2 Table. All the experiments were performed in the CFX96 Real-Time System (BIO-RAD).

Western blot analysis

Fly heads were collected at the indicated time points and homogenized with pestles, protein extracts were prepared with HEPES-Triton lysis buffer (1X HEPES-Triton buffer, 1 mM DTT, 0.4%NP-40, 0.1% SDS, 10% glycerol, 1X tablet protease inhibitor). Proteins were quantified using BSA assay. For immunoblot analysis, proteins were transferred to PVDF membranes (Genesee Scientific) and incubated with anti-DOM-A (1:300, GenScript), anti-DOM-B (1:5, from Dr. Peter B. Becker) and anti-ACTIN (1:100,DSHB) in blocking solution. Band intensity was calculated and analysed with Image J.

Statistics analysis

Statistical analysis of two data points was performed with Student's t-test. Statistical analysis of multiple data points was performed with one-way analysis of variance with Tukey post-hoc tests using GraphPad software.

Supporting information

S1 Fig. *Dom* RNAi efficiency. A. Schematic map of the *domA* and *domB* locus. Regions of the *domA* and *domB* mRNA targeted by the four specific RNAi lines. $dom^{RNAi\#1}$ and $dom^{RNAi\#2}$ *are* RNAi lines targeting different regions of *dom*. $domA^{RNAi}$ and $domB^{RNAi}$ are short hairpin RNAi lines specifically targeting *domA* or *domB* isoform CDS regions.
B. Quantitative RT-PCR showing the expression of *dom*, *domA* and *domB*. Flies were collected at ZT1. Downregulation of *dom* ($dom^{RNAi\#1}$ and dom^{RNAi2}) in circadian neurons decreased *dom* mRNA levels, comparing to the controls. Downregulation of *domA* and *domB* ($domA^{RNAi}$

and $domB^{RNAi}$) in circadian neurons specifically decreased *domA* and *domB* mRNA levels, comparing to the relative controls. Error bars correspond to SEM. n.s., nonsignificant; $^{**}p < 0.01$;$^{***}p < 0.001$;$^{****}p < 0.0001$ as determined by t-test.
(TIF)

S2 Fig. PER expression pattern in other groups of circadian neurons. A-B. Whole mount immunostaining showing the expression pattern of PER in LNds and DN1s. Red is PER and green is PDF. Flies were entrained for 4 days in LD and transferred to DD and dissected every 4 h on the fifth day. Downregulation of DOM decreased PER levels at CT1-9 and CT17 in LNds, while reduced PER levels at CT1-5 and CT17-21 in DN1s (Scale bar: LNds, 50 um; DN1s, 150 um).
(TIF)

S3 Fig. DOM-A, but not DOM-B was clk dependent. A-B. Quantitative RT-PCR showing the expression patterns of *domA* and *domB* in w^{1118} and Clk^{out} flies heads. Flies were collected at the indicated time points (ZT = Zeitgeber Time or CT = circadian time). *Dom-A* exhibited a strong oscillation pattern with a trough around ZT9 and CT9 while has a peak expression near ZT21 and CT21 in w^{1118} flies heads, while the oscillation of *dom-A* expression was abolished in Clk^{out}. There is no obvious oscillation for *dom-B* expression in w^{1118} and Clk^{out} flies heads both ZT and CT conditions.
C-D. Validation of specificity for *domA* and *domB* shRNA knockdowns and overexpression. *UAS-domA*, *UAS-domB* and *UAS-shRNA* of *domA* and *domB* males for domA and domB were crossed with tim-Gal4 females. F1 offspring adult brains are probed with anti-DOM-A and anti-DOM-B antibodies in western blot. ACTIN signals provided controls.
E-H. Western blot showing the expression patterns of DOM-A and DOM-B in w^{1118} and Clk^{out} flies heads. Flies were collected at the indicated time points (ZT = Zeitgeber Time). DOM-A did not show a strong oscillation pattern in w^{1118} flies heads, while the expression levels of DOM-A were remarkably decreased in Clk^{out}. There is no obvious change for DOM-B expression in w^{1118} and Clk^{out} flies heads. Band intensity was calculated and analyzed with the Image J. Error bars correspond to SEM. $^{*}P < 0.05$;$^{**}p < 0.01$,$^{***}p < 0.001$ as determined by the t-test.
(TIF)

S4 Fig. *DomA* and *domB* locomotor activity. A. Average locomotor activity of flies of different genotypes under 3 days of 12:12 hr LD conditions. Dark activity bars represent the night, and white bars represent the day. Comparing to the control (left panel),morning anticipation was severely disrupted in *domA* shRNAi lines (middle panel),while morning anticipation was normal in *domB* downregulation flies (Right panel).
B. Morning anticipation was calculated following the method described before. Error bars correspond to SEM. n.s., nonsignificant;$^{****}p < 0.0001$ as determined by t-test.
(TIF)

S5 Fig. Downregulation of DOM in adult stage decreases the mRNA levels of *per* and *tim*. **A-B.** Quantitative RT-PCR showing the expression of *per* and *tim*. Flies were collected at the indicated time points (ZT = Zeitgeber Time). Downregulation of *dom* in adult stage decreased *per* and *tim* mRNA levels.
(TIF)

S6 Fig. Constitutively activation of PDFR signaling rescued the TIM expression in sLNvs. **A.** Quantitative RT-PCR showing the expression of *dom*. Flies were collected at ZT1. Downregulation of *dom* ($dom^{RNAi\#1}$) in circadian neurons decreased *dom* mRNA levels (positive

control). *Dom* mRNA level is still reduced by *dom* RNAi even expressing the membrane-tethered PDF. Error bars correspond to SEM. $^{***}p < 0.001$;$^{****}p < 0.0001$ as determined by t-test. **B**. Representative confocal images of brains of *dom* RNAi flies expressing the membrane-tethered PDF or scrambled PDF. Flies were entrained for 4 days in LD 25˚C, and brains were dissected at ZT23 for anti-PDF antibody (green) and anti-TIM antibody (red). From top to bottom: (Top panel) fly brain expressing the membrane-tethered scrambled PDF; (middle panel) $dom^{RNAi\#1}$ flies expressing a membrane-tethered scrambled PDF; and (bottom panel) $dom^{RNAi\#1}$ flies expressing the membrane-tethered PDF. Confocal images are whole brain and soma of sLN_vs from left to the right (Scale bar: whole brain, 500 um; sLNvs, 50 um). **C.** Quantification of the number and relative PDF and TIM levels of sLNvs. For each genotype, totally, 14–20 flies brains and 30–55 neurons were used for quantification of the staining. Error bars correspond to SEM. n.s., nonsignificant;$^{***}p < 0.001$, $^{****}p < 0.0001$ as determined by t-test.
(TIF)

S1 Table. DOM regulates drosophila circadian behavior.
(PDF)

S2 Table. Primers used in this study.
(PDF)

Acknowledgments

We would like to thank Drs. Luoying Zhang, Patrick Emery, Pedro Miura, and Alexander M. van der Linden for comments and discussions on this manuscript. We thank all the members in the Zhang lab for technical support and helpful discussions. We thank Dr. Becker for the fly strains of *domA* and *domB* shRNA, as well as the DOM antibodies. We thank the Bloomington stock center for various fly stocks. We are grateful for TIM and PER antibodies kindly given by Dr. Rosbash and Dr. Stanewsky. We also thank the Developmental Studies Hybridoma Bank for PDF and GFP antibodies. We thank the Cellular and Molecular Imaging Core facility and Dr. Wei Yan's lab for equipment use. This work was initiated in Dr. Patrick Emery's lab.

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